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Genetic variation and relatedness in Tunisian wheat midges of the genus *Mayetiola* (Diptera: Cecidomyiidae), inferred from biological and molecular data

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Abstract: The Hessian fly *Mayetiola destructor* Say and the barley stem gall midge *Mayetiola hordei* Kieffer are two sibling phytophageous species causing, annually, important losses to cereals in Tunisia. Damage to wheat has usually been attributed to *Mayetiola destructor* but there is no strict association between the cereal species (wheat or barley) and the *Mayetiola* species (*destructor* or *hordei*). An effective pest management program requires an accurate analysis of the midges' genotypes. In the present study, we used the RAPD technique in conjunction with mating bioassays and mitochondrial DNA typing, to assess the extent of genetic variability and taxonomic relatedness, within a *Mayetiola* population infesting wheat in the North of Tunisia. Phylogenetic analysis inferred from the RAPD showed that the studied population had a variable genetic background that could be due to a complex epidemiological situation where wheat samples would be co-infested by both *M. destructor* and *M. hordei*. Although mating assays did not result in oviposition in a few cases (2/14), mitochondrial typing based on the Cyt b gene restriction analysis showed that all samples were *M. destructor* ones. Our results are additional evidence that RAPD variability is not a reliable criterion for taxonomical inference and provide support for allopatric distribution of *M. destructor* and *M. hordei* species, in Tunisia.

Key words: Diptera; *Mayetiola*; species; cytochrome b gene; RAPD; PCR-RFLP; mating

Dipterans of the *Mayetiola* genus are destructive pests that cause important losses on cereals. The insect's female lays 200 to 300 eggs on the leaves of infested plants (Ratcliffe and Hatchett, 1997). Upon hatching, larvae enter the leaf sheath and feed near the crown or at nodes, resulting in stunting of the plant and development of a dark green colour in the leaves (Beyers and Gallun, 1972; Zantoko and Schukle, 1999). The *Mayetiola* genus includes two phytophageous species, namely *Mayetiola destructor* Say (commonly known as the Hessian fly) and *Mayetiola hordei* Kieffer (commonly known as the barley stem gall midge).

There is evidence that *Mayetiola destructor* historically followed wheat from its original habitat in South-West Asia until it dispersed throughout most wheat-growing areas in the world (Briggle *et al.*, 1982). Nowadays, this species is found in Asia, Siberia, Europe, North Africa, North America and New Zealand (Ratcliffe and Hatchett, 1997). Wheat is the preferred host of the Hessian fly, although barley, rye, triticale and several volunteer grasses (such as *Agropyron* and *Aegilops*) may occasionally serve as hosts when wheat is not available (Buntin and Raymer, 1989; Ratcliffe and Hatchett, 1997).

Mayetiola hordei was mentioned only in North Africa and Southern Europe (Skuhrava *et al.*, 1984; Gagne *et al.*, 1991). It reproduces on barley but it can also sometimes be found on wheat (Mezghani *et al.*, 2002a). Thus, there is no strict association between the cereal species (wheat or barley) and the *Mayetiola* species (*destructor* or *hordei*).

The two phytophageous species of the *Mayetiola* genus can difficultly be distinguished morphologically. In fact, Gagne *et al.* (1991), using scanning electron microscopy to analyse midges from Morocco, reported differences in the shape of female postabdomen, the structure of male terminalia and the number of spicules covering the puparium. In Tunisia, Makni *et al.* (2000) identified diagnostic allozyme markers of taxonomic value for the two species; however, this technique requires refined conservation conditions to preserve allozyme activity.

More recently, Mezghani *et al.* (2002b)

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investigated the correlation between allozyme markers (Makni *et al*., 2000) and mitochondrial DNA sequence variation, by sequencing the cytochrome b (Cyt b) gene from a collection of Tunisian *M. destructor* and *M. hordei* samples. The authors reported three mitochondrial haplotypes, namely haplotype A found in *M. destructor* and haplotypes B and C found in *M. hordei*. Based on this sequencing data, it was possible to diagnose the two sibling species of *Mayetiola* by means of the Restriction Fragments Length Polymorphism (RFLP) of the Cyt b gene (800 bp). In fact, using the VspI endonuclease, Mezghani *et al*. (2002c) showed that haplotype A, in *M. destructor*, was represented by two restriction fragments (540 and 260 bp), whereas haplotypes B and C, in *M. hordei*, were represented, respectively, by three (540, 170 and 90 bp) and two (630 and 170 bp) restriction fragments. Although this is a simple and reliable approach, inferences regarding taxonomical specificity need to be further consolidated by conducting mating bioassays, according to the biological species concept (Mayr, 1974).

In Tunisia, widespread outbreaks of *Mayetiola*, causing extensive crop losses on cereals, have been reported since the early 1900's (Pagliano, 1935; Miller *et al*., 1989; Makni, 1993). Particularly on wheat, damage caused by these insects was estimated at 40% of durum wheat (*Triticum durum* Desf.) and 26% of bread wheat (*Triticum aestivum* L.) (Makni, 1993). Breeding Tunisian wheat varieties for genetic resistance to *Mayetiola* has been a major research focus in order to reduce yield losses (Bouktila *et al*., 2005). However, for this approach to succeed, *Mayetiola* populations occurring on wheat should be studied in terms of genetic variability and species identity. In this context, the Randomly Amplified Polymorphic DNA (RAPD) technique is a rapid and cost-effective methodology that has successfully been used to detect polymorphism in several insect species (Black *et al*., 1992; Vanlerberghe-Masutti and Chavigny, 1998; Naber *et al*., 2000).

Our objectives in the present study were: (i) to assess the extent of genetic variability within a *Mayetiola* population infesting wheat in one of the Tunisian cereal-growing regions and (ii) to clarify whether the genetic community of *Mayetiola* on wheat, in the studied region, is con-specific (all belonging to *M. destructor*) or made by a mixture of *M. destructor* and *M. hordei*.

1 MATERIALS AND METHODS

1.1 Insect material and rearing methods

A hundred wheat plants, infested with *Mayetiola* pupae, were randomly sampled in April 2004, from private fields located along a 5 kilometers Northwest / Southeast axis, across the region of Jedeida, 30 km to the Northwest of Tunis (Fig. 1). Those infested plants were transferred to the laboratory, inside plastic bags. The rearing method was similar to that described in Cartwright and Lahue (1944): *Mayetiola* larvae at the puparium stage were conserved inside cheesecloth-covered cages in a greenhouse where conditions are favourable for adult emergence (12 h photoperiod from 6:00 to 18:00, 20 ± 1℃ temperature and 60% relative humidity). On this regime, adults emerged from 5:30 to 8:30 (McKay and Hatchett, 1984). Thus, virgin adults were obtained readily by inspecting the cages at regular 2 min intervals from 5:30 to 8:30 and determining the sex. Eighteen adult insects having emerged in these conditions were used for RAPD analysis, whereas 28 virgin others (14 males and 14 females) were used for mating tests and subsequent PCR-RFLP analysis.

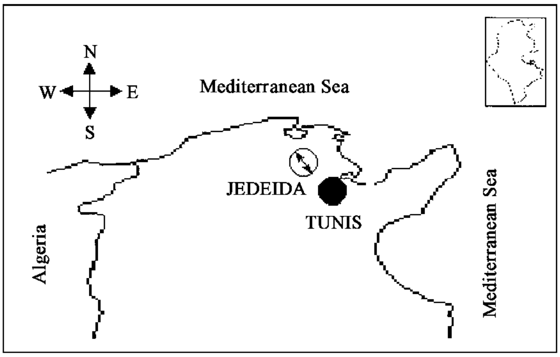


Fig. 1 A map showing the geographic location of the studied population of *Mayetiola* (open circle) and the sampling axis (arrow)

1.2 DNA isolation

For molecular analyses, we isolated DNA from each insect individual following the Doyle and Doyle (1987) DNA extraction protocol. Each fly was placed in a separate Eppendorf tube and ground with a miniature pestle in 200 μL of extraction buffer (100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 0.2% 2-mercaptoethanol). After incubation at 65 ℃ for 30 min, 200 μL of chloroform: isoamyl alcohol (24:1) were added, mixed and centrifuged at 8000 rpm for 5 min at 4 ℃ and the supernatant was transferred to a new tube. Nucleic acids were precipitated overnight with isopropanol (1:1) at −20 ℃. The mixture was centrifuged at 13500 rpm for 15 min at 4 ℃, the supernatant discarded and the remaining pellet washed in 70% Ethanol for 10 min and dissolved in 20 μL sterile water.

1.3 RAPD amplification

DNAs isolated from 18 *Mayetiola* individuals were amplified in a volume of 25 μL (Williams *et al*., 1990) with 25 ng of template genomic DNA, 1.75 units of Taq DNA Polymerase (QBiogene, France), 100 μM of dNTPs, 100 ng of primer and 2.5 μL of 10X reaction buffer supplied by the enzyme manufacturer. A set of 5 primers of arbitrary sequences, supplied by Operon Technologies (USA), was used for RAPD amplification (Table 1). Amplifications were performed in a thermal cycler "Crocodile Ⅲ" (Qbiogene, France) programmed as follows: 1 cycle of 5 min at 95 ℃; 35 cycles of 30 seconds at 95 ℃, 1 min at 35 ℃ and 1 min at 72 ℃; and a final cycle of 5 min at 72 ℃. A negative control without DNA was added in each run to test for contamination and each reaction was replicated twice to check for reproducibility of the RAPD patterns.

Table 1 Codes and sequences of primers used, total number of bands analysed and fragment size

Primer	Sequence	Total number of bands generated	Band size range (bp)
OPD08	5′-GTGACGTAGG-3′	22	250 – 1 800
OPD10	5′-GTGATCGCAG-3′	22	300 – 3 000
OPD15	5′-TTCCGAACCC-3′	19	220 – 2 800
OPD16	5′-AGCCAGCGAA-3′	30	220 – 2 500
OPD20	5′-GTTGCGATCC-3′	19	220 – 1 800
Total	–	112	220 – 3 000
Mean/primer	–	22.4	–

Amplification products were separated by electrophoresis in a 1.5% agarose gel in 0.5% TBE buffer. Amplification products were visualized under UV lights and photographed after staining the gel in a solution containing 1 μg/mL ethidium bromide. The molecular weight of each DNA band was estimated by comparing with a co-migrating 1 kb ladder.

1.4 RAPD data statistical analysis

Under the assumption that variation in banding patterns represents allelic segregation at independent loci, each RAPD band was treated as a two-allele system coded as 1 (presence of the band) or 0 (absence of the band). The extent of genetic variability within the studied population was estimated by 2 parameters: (i) the percentage of polymorphic markers; and (ii) the genetic distance (Nei and Li, 1979) calculated from the binary data for all pair-wise combinations of samples using the formula: "$D = 2n_{AB}/(2n_{AB} + n_A + n_B)$", where n_A is the number of bands present in individual A only, n_B the number of bands present in individual B only and n_{AB} the number of bands present in both A and B. Using this Nei and Li's (1979) distance, cluster analysis was performed to study genetic relationships between *Mayetiola* individuals. Two methods of cluster analysis, the neighbour-joining (NJ) (Saitou and Nei, 1987) and the unweighted paired-group method for the arithmetic average (UPGMA) (Sneath and Sokal, 1973), were used. The "RAPDistance Programs Version 2.00" (Armstrong *et al*., 1999) was used for genetic distance matrix calculation and NJ dendrogram construction, whereas the "PHYLIP package Version 3.572 C" (Felsenstein, 1995) was used for the UPGMA dendrogram construction.

1.5 Mating bioassays

The mating assays were carried out in a greenhouse set at 12 h photoperiod from 6:00 to 18:00, 20 ± 1 ℃ and 60% relative humidity. Five seeds of a *Mayetiola*-susceptible wheat cultivar, "BlueBoy" (kindly provided by the Kansas State University, Manhattan, USA), were planted in each of 14 soil-filled pots. In each pot, 1 virgin male and 1 virgin female of *Mayetiola*, having recently emerged from infested wheat samples collected from Jedeida, were released for mating inside a cheesecloth tent placed over the pot. After 48 h, tents were removed and the 28 dead adult flies were collected. Plants were examined individually under a microscope for eggs.

1.6 Mitochondrial DNA amplification

The 28 *Mayetiola* individuals used for mating tests were subjected to mitochondrial DNA amplification. An 800-bp DNA segment of the cytochrome b (Cyt b) gene was amplified using a couple of primers CP1 (5′-GATGATGAAATTTTGGATC-3′) and CP2 (5′-CTAATGCAATAACTCCTC-3′) (Mezghani *et al*., 2002c). Each PCR reaction mixture contained 10 ng of template DNA, 2.5 μL of 10X reaction buffer (Qbiogene, France), 1 unit of Taq DNA Polymerase (Qbiogene, France), 100 mM of dNTPs, 0.7 μM of each primer. The final reaction volume was 25 μL. Thermal-cycling was performed in a "Crocodile Ⅲ" PCR machine (Qbiogene, France), as follows: a cycle of 1 min at 95 ℃, 35 cycles of 1 min at 95 ℃, 1 min at 48 ℃ and 1 min at 72 ℃; and a final cycle of 5 min at 72 ℃.

1.7 Restriction analysis

15 μL of each PCR product were separately digested, overnight, by 2.5 U of the restriction endonuclease Vsp I (ATT, AAT) at 37 ℃ (Mezghani *et al*., 2002c). The digestion products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualised under UV lights.

2 RESULTS

2.1 RAPD analysis

112 different RAPD bands were generated by the 5 primers analysed. The total number of bands scored per primer ranged from 19 (OPD15 and OPD20) to 30 (OPD16) with an average of 22.4 bands per primer. The size of the amplified fragments ranged from 220 to 3 000 base pairs (bp). Table 1 summarises these data.

Many different patterns were detected within the 18 studied *Mayetiola* individuals from Jedeida population, indicating heterogeneity within the population. Fig. 2 shows an example of RAPD profile illustrating this variability. Among the 112 bands scored, 101 ones (90.1%) were polymorphic. Furthermore, the studied individuals showed differences in the levels of genetic distance, ranging from 0.06 to 0.63, with a mean genetic distance equalling 0.27 (Fig. 3). This indicated that the studied samples, within the Tunisian population of *Mayetiola* infesting wheat, had a variable genetic background.

Cluster analysis based on the genetic distances yielded two similar trees by both the UPGMA and the NJ methods (Fig. 4). Both analyses grouped the population into 2 distinct clusters: a major cluster with 15 (NJ) or 16 (UPGMA) individuals and a minor cluster with 3 (NJ) or 2 (UPGMA) individuals. The minor cluster individuals were outside the remaining *Mayetiola* samples, reflecting an evolutionary tendency, possibly with respect to their taxonomical specificity.

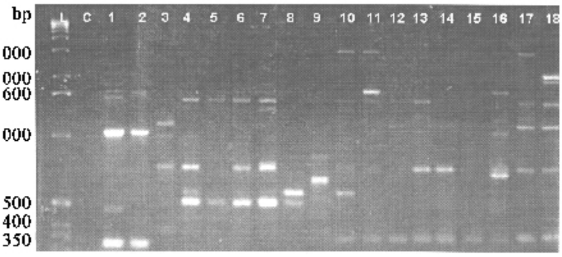
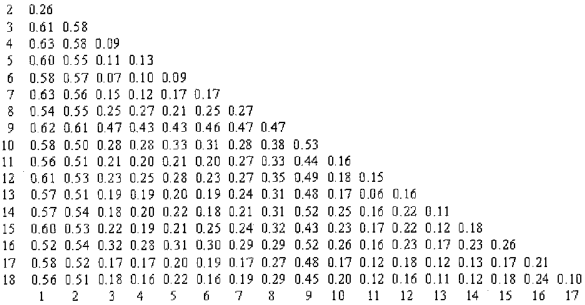


Fig. 2 RAPD pattern generated by using DNA from 18 *Mayetiola* individuals of the Jedeida population, with primer OPD10
L: Molecular size marker; C: Negative control without DNA.



2	0.26																
3	0.61	0.58															
4	0.63	0.58	0.09														
5	0.60	0.55	0.11	0.13													
6	0.58	0.57	0.07	0.10	0.09												
7	0.63	0.56	0.15	0.12	0.17	0.17											
8	0.54	0.55	0.25	0.27	0.21	0.25	0.27										
9	0.62	0.61	0.47	0.43	0.43	0.46	0.47	0.47									
10	0.58	0.50	0.28	0.28	0.33	0.31	0.28	0.38	0.53								
11	0.56	0.51	0.21	0.20	0.21	0.20	0.27	0.33	0.44	0.16							
12	0.61	0.53	0.23	0.25	0.28	0.23	0.27	0.35	0.49	0.18	0.15						
13	0.57	0.51	0.19	0.19	0.20	0.19	0.24	0.31	0.48	0.17	0.06	0.16					
14	0.57	0.54	0.18	0.20	0.22	0.18	0.21	0.31	0.52	0.25	0.16	0.22	0.11				
15	0.60	0.53	0.22	0.19	0.21	0.25	0.24	0.32	0.43	0.23	0.17	0.22	0.12	0.18			
16	0.52	0.54	0.32	0.28	0.31	0.30	0.29	0.29	0.52	0.26	0.16	0.23	0.17	0.23	0.26		
17	0.58	0.52	0.17	0.17	0.20	0.19	0.17	0.27	0.48	0.17	0.12	0.18	0.12	0.13	0.17	0.21	
18	0.56	0.51	0.18	0.16	0.22	0.16	0.19	0.29	0.45	0.20	0.12	0.16	0.11	0.12	0.18	0.24	0.10
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17

Fig. 3 Pair-wise genetic distances (Nei and Li, 1979) between 18 analysed *Mayetiola* individuals infesting wheat in Jedeida

2.2 Mating assays and cytochrome b gene polymorphism

Plants examination, 48 h after infestation, showed that wheat plants of the "BlueBoy" cultivar harboured *Mayetiola* eggs in 12 different assays, indicating a fecundity rate of 85.7% (12/14) within the studied *Mayetiola* samples having emerged from infested wheat in Jedeida.

The 14 pairs of *Mayetiola* used in the mating study were subjected to DNA isolation followed by amplification of the Cyt b gene and its restriction by Vsp I. This digestion yielded a uniform pattern consisting of two fragments sized 540 bp and 260 bp (Fig. 5), indicating that all the analysed individuals are *M. destructor* ones (haplotype A).

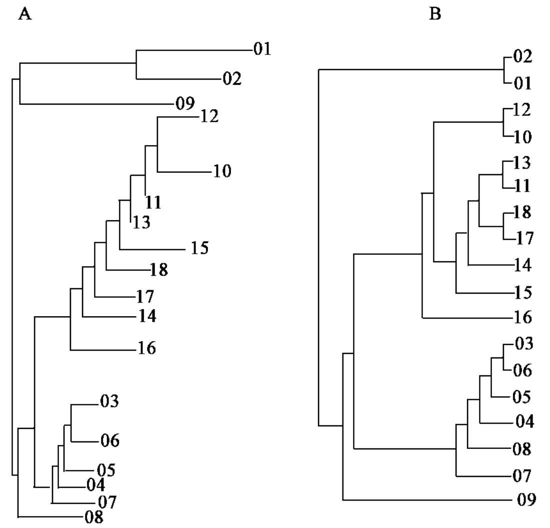


Fig. 4 Cluster analyses based on Nei and Li's (1979) genetic distance, showing genetic relationships between the 18 individuals of *Mayetiola* collected from wheat in Jedeida
A: Neighbour-Joining dendrogram; B: UPGMA dendrogram.

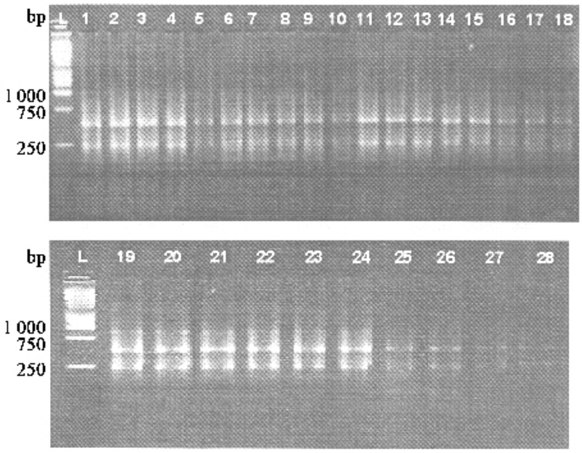

bp
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1 000
750
250
bp
L 19 20 21 22 23 24 25 26 27 28
1 000
750
250

Fig. 5 RFLP pattern of PCR-amplified cytochrome b (Cyt b) gene, digested by the restriction endonuclease VspI
L: Molecular size marker. Lanes 1 – 28: Digestion of Cyt b amplicons from the 28 *Mayetiola* individuals used in mating tests shows a uniform pattern corresponding to haplotype A of *Mayetiola destructor*.

3 DISCUSSION

The aim of this work was to study a *Mayetiola* population occurring on wheat in Jedeida (North Tunisia) in terms of genetic variability and species identity. Using the RAPD technique, we showed a substantial amount of variation in RAPD profiles among *Mayetiola* individuals，indicating a variable genetic background of insects from this genus that infest wheat plants. The dendrograms generated from the genetic distance matrix by the UPGMA and NJ methods showed only minor differences，between both methods，in the grouping of the *Mayetiola* samples，thus it was concluded that the analysed population derived from two founder groups：founder group 1 comprised the majority of analysed flies，whereas founder group 2 included a few number（3 by the NJ method and 2 by the UPGMA）. Previous studies have already shown that the RAPD technique can be an indictor for species identification（Guizani *et al*.，1994）. Thus，the high genetic heterogeneity within *Mayetiola* individuals infesting wheat，coupled with the uniqueness of a minor group of individuals in both cluster analyses could correspond to the existence of two sibling species of *Mayetiola* infesting wheat in Tunisia，namely *M. destructor*，having a natural preference for wheat，and *M. hordei*，which could occasionally reproduce on this plant（Mezghani *et al*.，2002a）.

In order to clarify whether the genetic community of *Mayetiola* on wheat，in the studied region，is conspecific（all belonging to *M. destructor*）or made by a mixture of *M. destructor* and *M. hordei*，we performed mating bioassays between 14 couples of *Mayetiola* having emerged from infested wheat in the same region. Although two out of the fourteen achieved assays did not result in oviposition，the mitochondrial typing of crossed insects indicated that they all belonged to *Mayetiola destructor* species.

Although the individuals were different from each other with respect to randomly amplified fragments profiles，they all belonged to a unique species，as inferred from the Cyt b gene restriction analysis. This comparison indicated that the RAPD profiles were not associated with the species trait，in the analysed population of *Mayetiola*. Indeed，inferences regarding taxonomical specificity and/or relatedness could not be ascertained by the RAPD（Guizani *et al*.，2002），as the mechanisms underlying product amplification remain obscure，due to the randomly nature of this technique（Williams *et al*.，1993；Mori *et al*.，1999）. Thus，the high extent of genetic diversity within the analysed *Mayetiola destructor* samples，estimated by the percentage of polymorphic bands and the Nei and Li's（1979）pair-wise genetic distances，would rather be attributed to：(i) the ancient colonization of Hessian fly on wheat in Tunisia，in a co-evolution process of the pest with its preferred host plant. In fact，*Mayetiola destructor* would have followed wheat from it's original habitat in South West Asia and dispersed to North Africa（Briggle *et al*.，1982；Naber *et al*.，2000）；and (ii) the sexual reproduction regime that makes the population both heterozygous and heterogeneous，contrarily to some insect species，such as aphids，were populations tend to constitute parthenogenetic clones（Puterka *et al*.，1993）.

The absence of eggs in two bioassays does not imply the existence of a mating barrier but is possibly the consequence of a non-preference for oviposition，conditioned by the plants vigour in the corresponding pots. Indeed，the plant genotype and vigour are essential factors that influence oviposition preference in many phytophageous insect species（Price，1994；Larsson *et al*.，1995）.

The results obtained in the present study raise the hypothesis that the two species of *M. destructor* and *M. hordei* would be separated geographically（allopatric）in Tunisia，with the presence of only *M. destructor* in Northern regions such as Jedeida，in association with extensive and mono-variety wheat culture. This suggests that *Mayetiola*-controlling efforts should be directed towards the resistance to *M. destructor* in the North of Tunisia and *M. hordei* in the South（Makni，1993）. A more extensive sampling will help study the insect populations occurring in Tunisia and analyse their genetic relationships.

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突尼斯两种麦瘿蚊的遗传变异和相关性分析

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摘要：小麦黑森瘿蚊 *Mayetiola destructor* Say 和大麦茎干瘿蚊 *M*. *hordei* Kieffer 是在突尼斯每年都可导致谷物重大损失的两个植食性姊妹种。通常认为为害小麦的瘿蚊是小麦黑森瘿蚊，但是不同谷类物种（小麦或大麦）与麦瘿蚊的这两个种（*destructor* 或 *hordei*）之间寄主关系并不很严格。提出有效的害虫管理方案首先要求对瘿蚊基因型进行精确分析。本研究应用随机扩增多态 DNA（RAPD）技术，结合交配分析和线粒体 DNA 分型技术，对位于突尼斯北部的一个为害小麦的麦瘿蚊种群的遗传变异程度和分类关联性进行了评估。基于 RAPD 结果的系统发育分析表明，所研究的种群具有较大的遗传变异范围，这可能源于被分析的小麦样品有被两种瘿蚊共同侵害的复杂背景。虽然交配分析表明有少数不能成功产卵（2/14），但是基于细胞色素 b 基因限制性酶切分析显示全部样品的线粒体分型均属 *M*. *destructor*。本文结果进一步证明以 RAPD 可变性作为分类推断依据不可靠，还为突尼斯 *M*. *destructor* 和 *M*. *hordei* 属于异域分布的观点提供了补充证据。

关键词：双翅目；麦瘿蚊属；物种；细胞色素 b 基因；PCR-RFLP；RAPD；交配

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